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Developmental Biology 306 (2007) 703–713
DEVELOPMENTAL BIOLOGY
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Early thalamocortical tract guidance and topographic sorting of thalamic projections requires LIM-homeodomain gene Lhx2

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Received for publication 15 July 2006; revised 6 April 2007; accepted 9 April 2007
Available online 14 April 2007

Abstract

The thalamocortical tract is the primary source of sensory information to the cerebral cortex, but the mechanisms regulating its pathfinding are not completely understood. LIM-homeodomain (LIM–HD) gene *Lhx2* has been proposed to participate in a combinatorial "code" to regulate dorsal thalamic patterning and also the topography of thalamocortical projections. Here, we report that *Lhx2*−/− embryos exhibit a gross disruption in the *early* development of the thalamocortical tract, such that thalamic axons are unable to enter the ventral telencephalon. A possible cause for this deficit is a severe reduction of "pioneer" cells in the mutant ventral telencephalon that constitutes a putative mechanism for guiding the entry of the thalamocortical tract into this structure *in vivo*.

However, *in vitro*, the thalamocortical tract is able to enter the ventral telencephalon, and this permitted an examination of whether thalamocortical topography is normal in the *Lhx2* mutant. Contrary to hypotheses that proposed a cell-autonomous role for Lhx2 in the thalamus, *Lhx2*−/− thalamic explants generate a normal topography of projections in control ventral telencephalic preparations. This is consistent with our findings of normal patterning of the *Lhx2* mutant dorsal thalamus using a wide array of markers. In the reverse experiment, however, control thalamic explants display aberrant topography in *Lhx2*−/− telencephalic preparations. This perturbation is restricted to projections from caudal thalamic explants, while rostral and middle explants project normally.

Thus Lhx2 is required for multiple steps in thalamocortical tract pathfinding, but these functions appear localized in the ventral telencephalon rather than in the dorsal thalamic neurons. Furthermore, the absence of Lhx2 in the ventral telencephalon selectively disrupts a subset of thalamic axon topography, indicating a specific rather than a general perturbation of cues in this structure.

Keywords: Lhx2; Thalamocortical; Specificity; Pathfinding; Topography; Sema6A; Internal capsule

Introduction

All sensory information, except olfaction, reaches the cerebral cortex via the thalamocortical tract, making it one of the major axonal tracts in the forebrain. Dorsal thalamic cells extend axons through the ventral thalamus, which cross the diencephalic–telencephalic boundary (DTB), and traverse through the ventral telencephalon, before they innervate the cerebral cortex in an area-specific manner. The sorting of thalamic projections in the ventral telencephalon is thought to presage the inter-areal topography of these projections when they innervate the cortex (Garel et al., 2002). The mechanisms that regulate proper navigation of thalamic axons along this complex route are not well understood.

A few transcription factors that are specifically expressed in the dorsal thalamus at early embryonic stages are leading candidates proposed to regulate multiple aspects of thalamocortical pathfinding. Specifically, the expression of *Lhx2*, *Lhx9*, *Ngn2* and *Gbx2* parcellates nuclei in the developing thalamus, suggesting differential cell-autonomous functions directing thalamocortical tract development (Nakagawa and O'Leary, 2001). Indeed, mice in which either *Ngn2* or *Gbx2* is disrupted have abnormalities in the thalamocortical tract. In the *Ngn2* mutant, there is a defect in the topography of projections from the rostral thalamus (Seibt et al., 2003), while in the absence of Gbx2, thalamic projections do not reach the cortex (Miyashita-

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doi:10.1016/j.ydbio.2007.04.007

Lin et al., 1999). The molecular mechanisms by which these transcription factors act are unclear, however. There are suggestions of a 'code' such that a combinatorial expression of the transcription factors *Lhx2*, *Lhx9*, *Ngn2* and *Gbx2* in distinct dorsal thalamic nuclei could regulate their identity and also the topography of their projections (Nakagawa and O'Leary, 2001; Bachy et al., 2002). This hypothesis has strong parallels in the spinal cord, where an "LIM code" i.e. combinations of LIM–HD genes specify distinct classes of motor neurons (Shirasaki and Pfaff, 2002) and also control their pathfinding (Kania and Jessell, 2003). Remarkably, the combinatorial action of LIM genes appears to be a conserved mechanism since an 'LIM code' is also seen in the *Drosophila* ventral nerve cord, where motor neuron target specificity is cell-autonomously controlled by a combinatorial expression of LIM genes (Thor et al., 1999). The *Drosophila* homolog of Lhx2, Apterous, regulates the trajectory of another subset of ventral nerve cord neurons (Lundgren et al., 1995). In zebrafish, *belladonna*/*lhx2* is required for axon guidance of forebrain commissures (Seth et al., 2006). In the mouse, *Lhx2* and *Lhx9* are both expressed in the dorsal thalamus from the earliest stages of its development, making them strong candidates for specification of cell identity as well as projection patterns of the dorsal thalamus.

Despite strong evidence for a role for LIM–HD transcription factors in axon pathfinding, no LIM–HD gene has yet been examined for such a role in the mammalian forebrain. In this study, we find that *Lhx2* mutant mice show multiple, distinct defects in the development of the thalamocortical pathway. The first defect prevents the entry of this tract into the ventral telencephalon *in vivo*. To examine later steps in the development of the thalamocortical pathway, we used an *in vitro* assay (Seibt et al., 2003), in which the thalamocortical tract is able to enter the ventral telencephalon. In this assay, we found a specific requirement for Lhx2 in establishing the topography of projections from the caudal, but not the rostral or middle thalamus. Concomitantly, expression of *Sema6A*, a gene known to regulate thalamocortical topography (Leighton et al., 2001), is aberrantly upregulated. This places Lhx2 among the few players thus far known to control the topography of thalamocortical pathfinding.

Materials and methods

Obtaining embryos

Lhx2+/− male and female mice (Porter et al., 1997) were crossed, and the day of plug was taken as embryonic day (E) 0.5. *Lhx2*−/− embryos die *in utero* by E15.5, limiting the range of ages available for analysis. For GFP-expressing control and Lhx2 mutant embryos, GFP-expressing mice (Jaxlabs, FVB.Cg-Tg (GFPU)5Nagy) were crossed into our *Lhx2*+/− breeding colony.

In situ hybridization

In situ hybridization was performed as previously reported (Vyas et al., 2003).

DiI placement

For thalamocortical tract labeling, E13.5–E14.5 control and *Lhx2* mutant embryos were harvested, the brains dissected out, and fixed overnight in 4% paraformaldehyde. The brains were cut down the midline, a small crystal of DiI (Molecular Probes) was placed in the dorsal thalamus, and the tissue was stored in 4% PFA at 37 °C for 2–3 weeks. The thalamocortical tract was imaged in 100 μm vibratome sections. For backlabeling IC pioneers, DiI placements were done at E12.5 and incubated 2–3 weeks. 100 μm sections were prepared using a vibratome and imaged using a confocal microscope. For the mutants, typically only one or two 100 μm sections contained any cells in the IC region, whereas in control brains, several 100 μm sections showed labeling in IC region. The 100 μm section(s) with the maximum staining in the IC region were selected for confocal imaging. Optical sections (layers) of 2 μm each were imaged through each 50 μm section and all layers with cells were stacked to give a composite.

In vitro cultures

This assay was set up following the protocol established by Seibt et al. (2003). Briefly, E14.5 control and *Lhx2*−/− embryos were harvested that were either GFP positive or negative. GFP positive control and mutant thalamus were sliced at 250 μm using a McIlwain tissue chopper. Each slice of GFP positive rostral, middle, or caudal dorsal thalamus was juxtaposed to a GFP negative telencephalic hemisphere prepared as in the schematic (Fig. 5) and placed on a 0.4 μm culture plate insert (Millipore). The cultures were fixed after 3 days *in vitro* and processed for anti-GFP immunohistochemistry as in Seibt et al. (2003), using anti-GFP rabbit polyclonal at 1:1000 dilution and Alexa-568 labeled goat anti-rabbit at 1:1000 dilution (Molecular Probes).

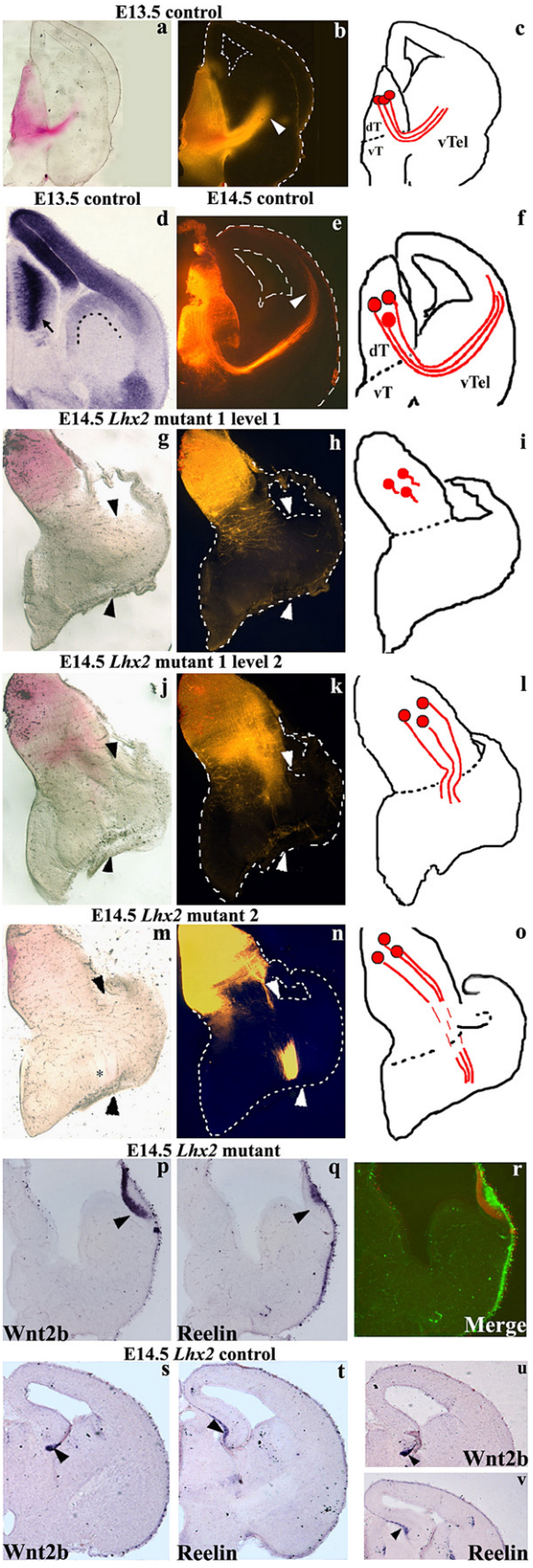
Quantitation of fluorescence

This was performed as in Seibt et al. (2003). Briefly, images were divided into 48 equal sized bins along the rostrocaudal axis of the explant. Using ImageJ software (NIH image), the average fluorescence intensity per bin was measured. To normalize for variations due to background between explants, for each explant, the bin with the maximum average intensity was given a value = 1. The intensity values for each bin of a given explant were represented as a fraction of the value of this maximum intensity bin. Statistical analysis (two-way ANOVA) was performed using the GraphPad Prism software.

Results

The thalamocortical tract originates in the dorsal thalamus and takes a complex route before it reaches the cortex. Each step in this route has been well characterized and occurs with a precise timing (reviewed in Garel and Rubenstein, 2004). The first step is growth into the ventral thalamus, in which the thalamocortical tract crosses the DTB and enters the ventral telencephalon, by E13.5 (Lopez-Bendito et al., 2006; Fig. 1b). Here, it makes a characteristic "v-shaped" turn dorsally, towards the boundary between the ventral telencephalon and the cortex (pallium–subpallium boundary, PSB). Finally, entry into the cortex occurs as early as E16 in the rat and E14 in the mouse (Catalano et al., 1991; Bicknese et al., 1994; Molnar et al., 1998a,b; Fig. 1e).

Lhx2 is expressed in the dorsal thalamus from early stages, suggestive of a cell-autonomous role in patterning this structure (Fig. 1d, arrow). *Lhx2* is also expressed in the entire telencephalic ventricular zone, appropriately positioned to function in precursors of ventral telencephalic cells and in the cortex (Bulchand et al., 2001; Fig. 1d, dotted line). To test whether Lhx2 is required in thalamocortical pathfinding, DiI crystals were placed in the dorsal thalamus of control and *Lhx2*−/− brains. By E13.5, control thalamic axons are labeled in a characteristic path through the internal capsule region of the ventral telencephalon, and by E14.5 they have reached the



cortex ($n=6/6$; Figs. 1b, e). *Lhx2*−/− axons, however, fail to cross the DTB and enter the ventral telencephalon by E14.5, instead growing ventrally within the thalamus ($n=9/9$; Figs. 1h, k, n). This is in spite of a distinct physical continuity between the thalamus and the ventral telencephalon that is seen in brightfield images of corresponding mutant sections (Figs. 1g, j, m). The neocortex does not develop in the absence of Lhx2, though some vestigial tissue is present in the dorsal half of the mutant telencephalon. This tissue consists of an expanded cortical hem, and a superficial layer of cells that are preplate cells, while the proper cortical plate fails to develop (Bulchand et al., 2001; Monuki et al., 2001). A cortical hem marker, *Wnt2b*, and *reelin* expression are seen in this residual tissue at E14.5, indicating that it does not represent residual neocortex (Figs. 1p–r). Since only neocortex sends projections directly to the dorsal thalamus, it is unlikely that any "corticothalamic" axons are present in the Lhx2 mutant.

To probe the nature of the early pathfinding defect in the *Lhx2* mutant, we examined several markers of the dorsal and ventral thalamus at two stages, E12.5, when the thalamocortical tract is just beginning to grow within the thalamus, and E14.5 when the tract has entered the cortex. These stages therefore span the duration in which the thalamocortical tract traverses the ventral telencephalon. We examined an array of markers, specifically including transcription factors known or proposed to play a cell-autonomous role in thalamocortical tract pathfinding: *Ngn2* (Seibt et al., 2003), *Gbx2* (Hevner et al., 2002), *Pax6* (Jones et al., 2002; Pratt et al., 2002), *Lhx2*, and *Lhx9* (Nakagawa and O'Leary, 2001). We find normal expression of these and other markers such as *Dbx1* in the *Lhx2*−/− dorsal thalamus at different rostrocaudal levels (Fig. 2). In particular, details of the expression patterns such as exclusion of the ventricular zone by *Gbx2* (Figs. 2a–f), and complementarity between *Ngn2* and *Lhx9* at caudal levels (Figs. 2q′, r′, w′, x′) are also preserved in the mutant. Additional markers that exhibit regional specificity and distinguish discrete populations within the thalamus such as *shh* in the zli (not shown), *Netrin 1*, and *Steel*, as well as markers that are specific to the ventral thalamus, such as *Dlx2*, *Isl1* (not shown), and *Lhx1*, exhibit normal patterns (Figs. 2a″–f″).

Fig. 1. The thalamocortical tract phenotype the *Lhx2* mutant. The thalamocortical tract enters the ventral telencephalon at E13.5 in control brains (arrowhead, b). By E14.5, thalamocortical axons have reached the cortex (arrowhead, e). *Lhx2* expression at E13.5 (d) is seen in the dorsal thalamus (arrow) and the telencephalic ventricular zone (black dashed line). In the *Lhx2*−/− brain, thalamocortical projections fail to enter the ventral telencephalon by E14.5 (h, k, n). Two levels of sections from the same mutant are shown in panels g–l. Panels m–o represent a different mutant brain, in which the thalamocortical axons grow ventrally within the thalamus. Panels a, g, j and m are brightfield images of the corresponding DiI images (b, h, k and n) respectively. Panels c, f, i, l and o are schematized illustrations of the adjacent DiI images. The DTB is marked by two arrowheads in panels g, h, j, k, m and n. Wnt2b marks the cortical hem (arrowhead, s, u) and Reelin is expressed in the marginal zone (arrowhead, t, v) of control brains at E14.5. (u and v) are higher magnification images of (s and t) respectively. In the *Lhx2* mutant, an expanded cortical hem is seen with a thickened layer of Reelin expression in marginal zone above it (arrowheads, p, q). A false-color overlay of panels p and q is shown in panel r.

In summary, no aberrant or ectopic expression of any marker is seen in the dorsal thalamus nor is there any marker missing that normally labels this structure. These observations suggest that the *Lhx2*−/− dorsal thalamus is patterned normally at early stages up to E14.5.

The ventral telencephalon is crucial in establishing the first steps of the thalamocortical pathway as an "intermediate target" before the tract enters the cortex (Metin and Godement, 1996; Tuttle et al., 1999; Garel et al., 2002; Lopez-Bendito et al., 2002; Yun et al., 2003). A newly discovered cellular population, the "corridor cells," has been identified to play a crucial role in thalamocortical guidance by creating a permissive channel for growth in otherwise non-permissive ventral telencephalic territory (Lopez-Bendito et al., 2006). Corridor cells are required not only *in vivo*, but also *in vitro*, to enable the growth of thalamocortical axons within the ventral telencephalon (Lopez-Bendito et al., 2006). We therefore examined markers of the corridor cells, *Ebf1* and *Isl1*, and also *Lhx6*, whose expression excludes the corridor (Lopez-Bendito et al., 2006). These markers show a similar pattern in the *Lhx2* mutant (Figs. 3a–d; g, h). This suggests that the corridor is normal in the *Lhx2* mutant. Furthermore, the corridor cells have not been shown to aid the crossing of the DTB since the explant culture assay used to demonstrate the requirement of the corridor cells did not test this particular feature. Rather, explants of dorsal thalamus were placed in ectopic ventral locations of the host explant from where they navigated successfully if a normal corridor was present (Lopez-Bendito et al., 2006).

A different cellular mechanism was proposed to aid the entry of the thalamocortical tract into the ventral telencephalon, though evidence in this regard is only correlative thus far (see Discussion). A population of neurons called "IC cells" was identified in the internal capsule region, which sends projections into the dorsal thalamus *prior* to thalamocortical axon outgrowth (Molnar and Cordery, 1999). These projections were proposed to pioneer the ingrowth of dorsal thalamic axons across the DTB and into the ventral telencephalon (Mitrofanis and Guillery, 1993; Molnar and Cordery, 1999). Definitive markers of this population have yet to be identified, but *Nkx2.1* expression marks some scattered cells in the IC region. This scattered labeling of *Nkx2.1* is thought to include the IC cell population, which is missing in the *Mash1* mutant, and suggested to be the cause of a thalamocortical entry-defect similar to what we report in the *Lhx2* mutant (Tuttle et al., 1999). *Nkx2.1* only serves as a marker for these cells, but is not crucial for their function, since thalamocortical pathfinding is normal in the *Nkx2.1* mutant (Marin et al., 2002). Regardless,

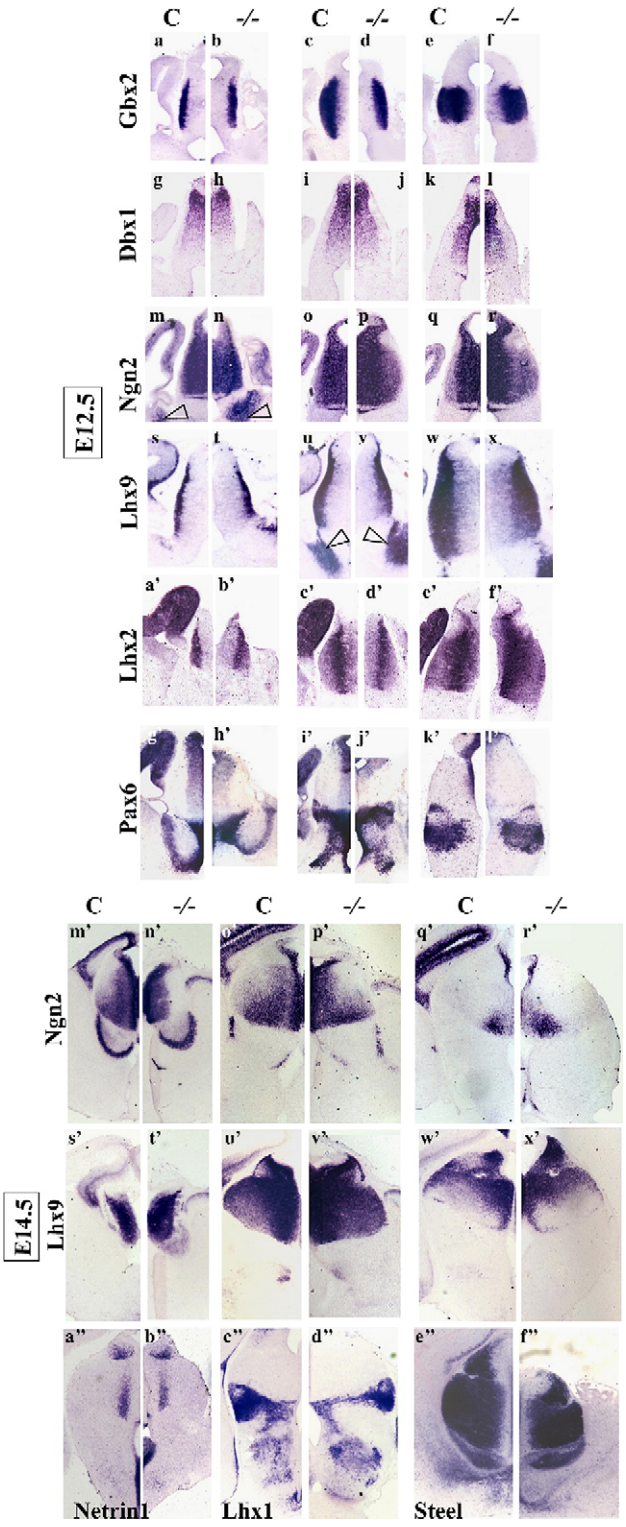


Fig. 2. Patterning of the thalamus in the absence of Lhx2. At E12.5 (a–k′), *Gbx2* (a–f) and *Lhx9* (s–x) expression is seen to largely exclude the ventricular zone and strongly label the mantle layer of the dorsal thalamus at rostral, middle, and caudal levels in control and mutant brains. *Ngn2* (m–r) and *Lhx2* (a′–f′) also discriminate between the ventricular zone and mantle by displaying different levels of expression in these regions, with *Ngn2* being stronger in the ventricular zone, and *Lhx2* in part of the mantle layer. *Dbx1* expression labels the ventricular zone of the dorsal thalamus selectively at all levels (g–l). *Pax6* expression is seen in a dorso-ventral gradient in the ventricular zone of the dorsal thalamus, and also in specific regions of the ventral thalamus in control and mutant sections (g′–l′). Open arrowheads in panels m, n, u and v mark expression of *Ngn2* and *Lhx9* in the thalamic eminence of control and mutant brains. At E14.5 (m′–f″), *Ngn2* (m′–r′) and *Lhx9* (s′–x′) expression in the dorsal thalamus displays similar patterns at all levels in control and mutant sections, specifically excluding or marking selective regions of the structure. *Netrin1*, *Lhx1* and *Steel* also show a region-specific expression in the control and mutant dorsal and ventral thalamus, shown at a single representative level for each marker (a″–f″). Notably, no aberrant or ectopic expression of any marker is seen in the dorsal thalamus nor is there any marker missing that normally labels this structure.

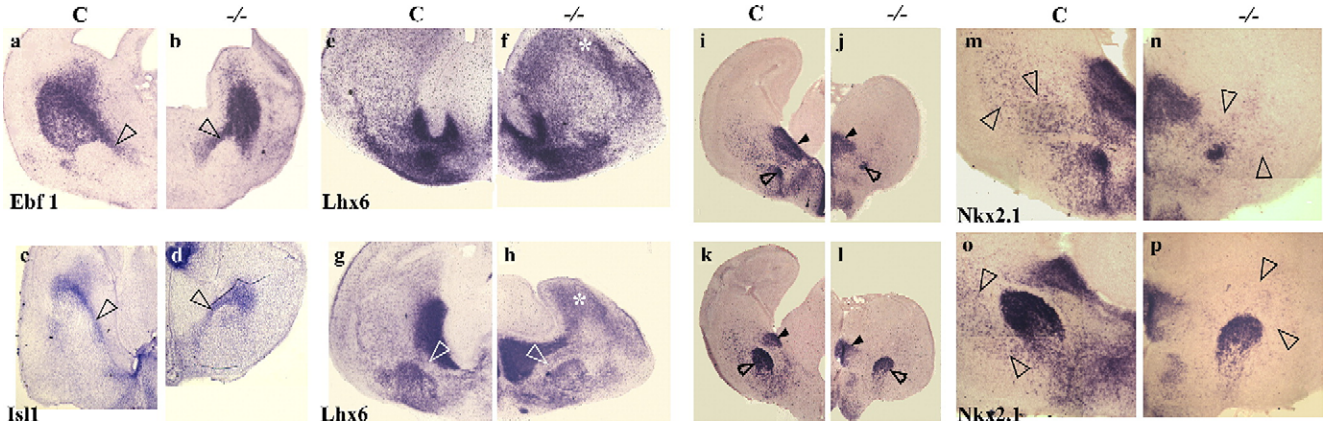


Fig. 3. Ventral telencephalic patterning in the absence of Lhx2. At E14.5, *Ebf1* and *Isl1* expression identifies corridor cells, while *Lhx6* expression excludes the corridor, but is present in domains on either side, in both control and mutant brains (open arrowheads, a–d, g, h). *Lhx6* expression is seen strongly in the MGE, and in scattered migrating interneurons all over the sections at rostral (e, f) and mid (g, h) levels of sectioning. There is also an apparently increased expression in the LGE of the mutant (asterisk, f, h). *Nkx2.1* expression marks the globus pallidus (open arrowheads, i, j, k, l), the MGE (black arrowheads, i, j, k, l), as well as scattered cells in the ventral telencephalon (open arrowheads m, n, o, p) of both control and *Lhx2* mutant brains. In the mutant, however, these scattered cells are greatly reduced in number (open arrowheads, n, p). Panels m–p are composite high magnification images of (i, j, k and l) respectively.

the expression of *Nkx2.1* suggested that there may be subtle deficits in the ventral telencephalon of the *Lhx2* mutant. Normally, this marker is expressed in the medial ganglionic eminence (MGE) at E12.5, and at E14.5 is seen in the globus pallidus and in residual MGE tissue. *Nkx2.1* is also seen in scattered cells in the ventral telencephalon (Fig. 3; Tuttle et al., 1999). In the *Lhx2* mutant, there appears to be a reduced number of these scattered *Nkx2.1*-positive cells, when examined across several rostrocaudal levels (Figs. 3i–p). Though we have previously reported the *Lhx2* ventral telencephalon to be grossly normal with respect to patterning (Bulchand et al., 2001), we have also identified a deficit within the amygdaloid complex: the nLOT2, one particular amygdaloid nucleus, is missing, while several other components of the amygdaloid complex are present (Remedios et al., 2004). The reduced number of *Nkx2.1* expressing cells at E14.5 appears to be another such specific deficit in the *Lhx2* mutant ventral telencephalon and motivates an examination of IC cells at earlier stages, E12.5, when the thalamocortical tract has yet to enter the ventral telencephalon. At this stage, however, no scattered *Nkx2.1* staining was seen in control brains (data not shown), suggesting that if *Nkx2.1* expression does indeed label IC cells, then it must appear only at later stages and is not useful as an early marker.

In search of additional markers for IC cells we examined *Lhx6* in detail since it too appeared to label scattered cells in the ventral telencephalon. However, consistent with the expression of *Lhx6* in MGE-derived interneurons (Lavdas et al., 1999), there is scattered labeling throughout the ventral telencephalon at different levels of both control and mutant sections. In fact, there appears to be an increase in labeling in the lateral ganglionic eminence (LGE) of the *Lhx2* mutant (asterisk, Figs. 3f, h), consistent with the possibility that there are more interneurons present since there is no cortex for them to migrate into. Therefore we focused on the only available definitive means of identifying IC cells, backlabeling them with DiI placements in the dorsal thalamus (Molnar and Cordery, 1999; Tuttle et al., 1999). Using similar procedures, we were able to identify a robust population of backlabeled IC cells in control E12.5 brains that were detected in several 100 μm sections when observed under epifluorescence ($n=2$; Fig. 4a). Confocal imaging of these sections was performed using 2 μm as the thickness of each optical section (layer). In control brain sections, a single 2 μm layer reveals numerous cells, and a complete stack of all layers shows a dense cluster of cells that cannot be resolved from each other (Fig. 4 shows stacks (c, d) and single layers (g, h) from two different control brains). In striking contrast, backlabeling experiments in the *Lhx2* mutant reveal an extremely small population of IC cells, limited to only one or two 100 μm sections in each brain ($n=6$; Fig. 4b). When imaged using a confocal microscope, only a few scattered cells are seen in a collapsed stack consisting of all layers containing labeled cells. This effect is highly reproducible since six different mutant brains consistently showed a massive reduction in the number of backlabeled IC cells, compared with control brains (Figs. 4e, f, i, j show complete stacks from four different mutant brains). These data suggest that there is a profound loss of backlabeled IC cells in the mutant brains. It is possible that more cells are present, but unable to project to the dorsal thalamus so that only a few cells are backlabeled in the mutant. The severe depletion of these hypothesized "pioneering" projections presents a possible basis for the inability of dorsal thalamic axons to enter the ventral telencephalon in the *Lhx2* mutant. Thus, the *Lhx2* mutant provides another piece of correlative evidence that links backlabeled IC cells to defects in the entry of the thalamocortical tract into the ventral telencephalon. An alternative possibility which remains is a cell-autonomous requirement for Lhx2 in thalamic neurons to enable them to cross the DTB, which can be tested when conditional mutants for this gene become available.

Based on its expression in the dorsal thalamus and the role of LIM–HD genes in axon pathfinding in other systems, Lhx2 was hypothesized to regulate the topographic mapping of thalamocortical axons (Nakagawa and O'Leary, 2001; Bachy et al.,

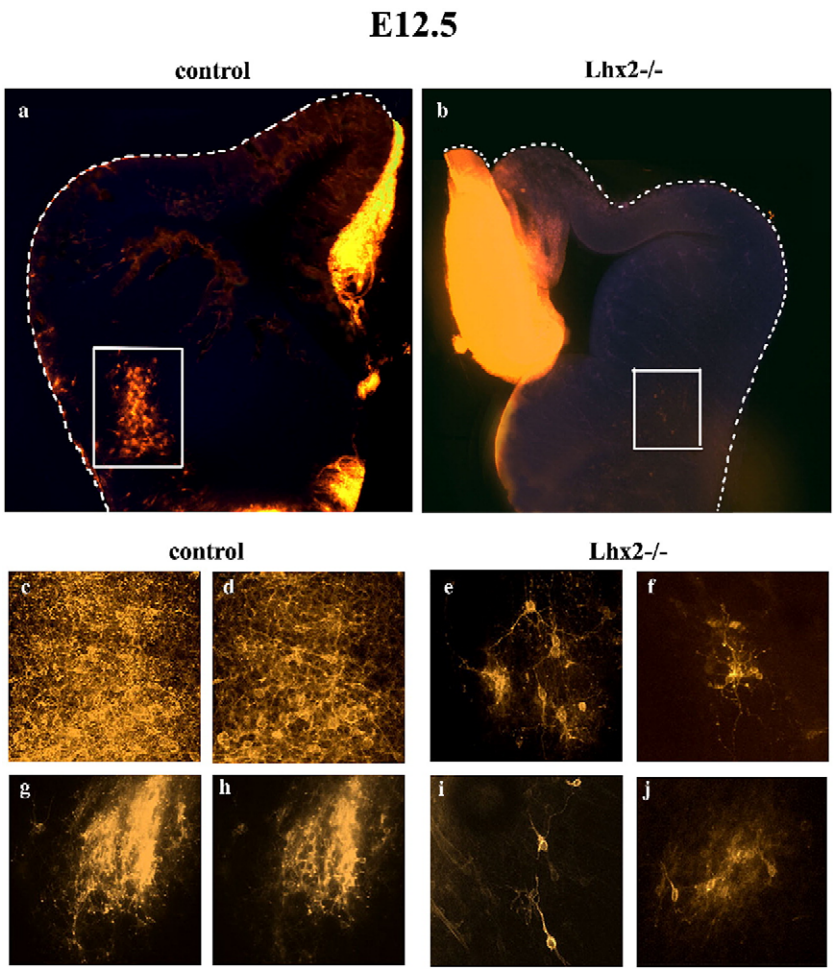


Fig. 4. IC cells in the ventral telencephalon of Lhx2 mutant brains. DiI placements at E12.5 mark IC pioneer cells in the ventral telencephalon of control brains (a), but these cells are not seen at low magnification in *Lhx2* mutant brains (b). 100 μm sections from two different control brains were selected for confocal imaging (c, g). Numerous cells are seen in a stack of all optical sections (2 μm layers) that contained cells (c=23 layers; g=23 layers), as well as in a single 2 μm layer from each stack (d, h). Confocal imaging of sections from four different *Lhx2* mutant brains (e, f, i, j) reveals very few cells in stacks of all layers that contained labeling (e=51; f=60; i=17; j=30 layers).

2002; Seth et al., 2006). The failure of the thalamocortical tract to enter the ventral telencephalon in the *Lhx2* mutant prevents testing of this hypothesis. We attempted to circumvent this problem *in vitro*. We reasoned that the complicated morphology of the thalamus–telencephalon junction may make crossing the DTB a particular challenge *in vivo*, but may not present such a difficult obstacle if the dorsal thalamus were juxtaposed directly to the ventral telencephalon *in vitro*, by removing the presence of the intervening ventral thalamus. An *in vitro* assay has been developed that uses precisely these tissues, and recapitulates aspects of the topographic organization of dorsal thalamic projections (Seibt et al., 2003). In this assay, open telencephalic preparations are juxtaposed to GFP-expressing rostral, middle, or caudal dorsal thalamic explants (schematic, Fig. 5). After 3 days *in vitro*, thalamic projections display topographic sorting, such that they grow in rostral, middle, and caudal directions within the ventral telencephalon, respectively (Seibt et al., 2003; Figs. 5a, d, g). We asked if *Lhx2* mutant thalamic axons are capable of growing into control ventral telencephalic preparations, and if so, whether they do so in a topographic manner. As in Seibt et al. (2003), E14.5 GFP-expressing control and *Lhx2*−/− dorsal thalamic explants were prepared, divided into rostral, middle, and caudal groups, and juxtaposed to telencephalic preparations from GFP-negative control embryos. After 3 days *in vitro*, explants were processed for GFP immunohistochemistry, revealing the growth of thalamocortical fibers. These were imaged and subjected to quantitative analysis. Images were divided into 48 bins and the average fluorescence intensity per bin was used as a measure of axonal growth (schematic, Fig. 5). Two-way ANOVA between control and mutant thalamic explants demonstrated no significant difference in axonal trajectories between the respective rostral, middle, and caudal groups (Figs. 5c, f, i). These results indicate that the *Lhx2*−/− thalamus can generate a topographic mapping comparable to that of control explants, when juxtaposed to control telencephalic tissue.

In the reverse experiment, when control thalamic tissue was juxtaposed to *Lhx2*−/− telencephalic preparations, rostral and middle thalamic explants generated a normal topography of projections in the mutant ventral telencephalon (Fig. 6). In

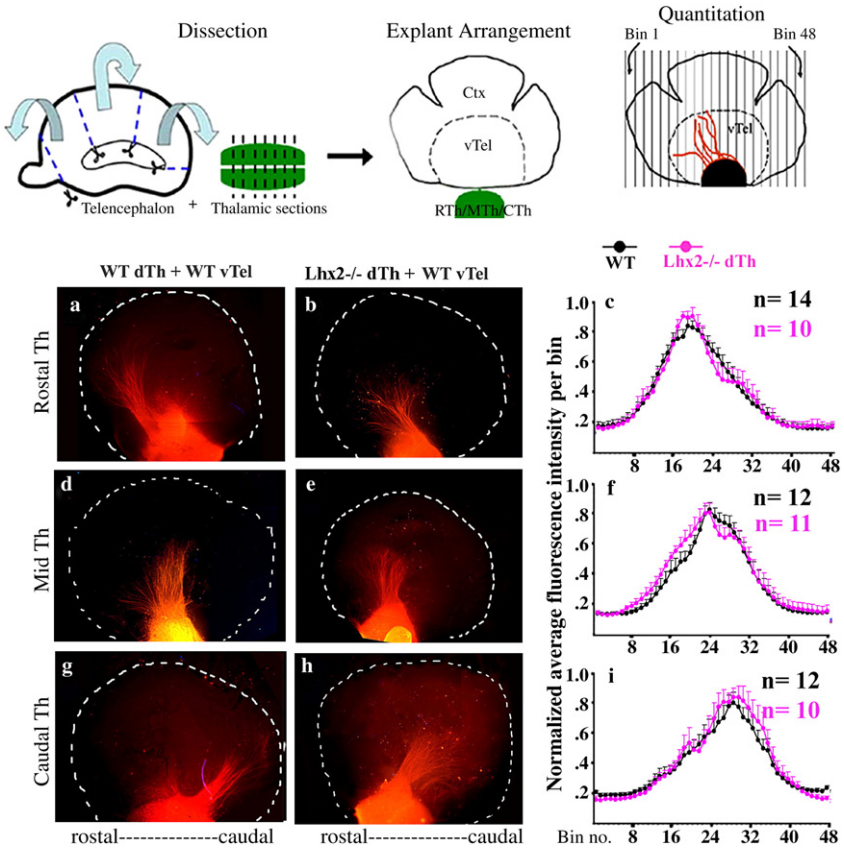


Fig. 5. Lhx2 mutant thalamic axons display normal topography *in vitro*. Organotypic explant culture preparations were set up as shown in the schematic. GFP-expressing thalamic explants from E14.5 control (a, d, g) or *Lhx2* mutant (b, e, h) dorsal thalamus were juxtaposed to control telencephalic explants (the contour of the explant is marked by dashed lines). The respective rostral (a, b), middle (d, e) and caudal (g, h) explants projected normally to corresponding regions in the ventral telencephalon. Quantitative representation of the fluorescence intensity across the explant reveals similar curves for control and *Lhx2* mutant thalamic preparations (c, f, i).

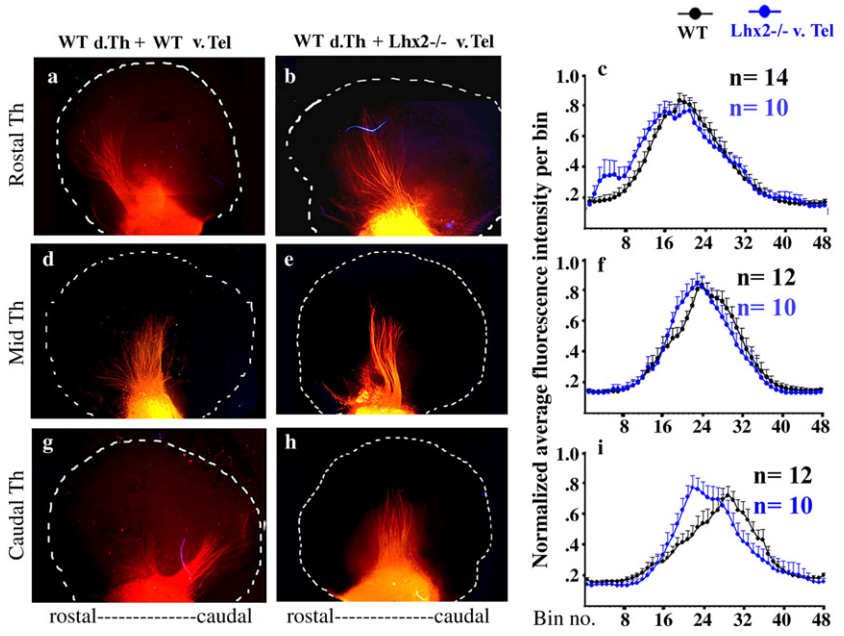


Fig. 6. Aberrant topography of thalamic projections into Lhx2 mutant ventral telencephalon. Organotypic cultures were set up as described for Fig. 5. Control rostral (b), middle (e) and thalamic explants juxtaposed to *Lhx2*−/− telencephalon revealed normal topographic projections. Control caudal thalamic explants failed to project normally, but grew in a medialized direction (h). Quantitative representations of pooled data indicate normal growth of rostral and middle preparations (c, f), but a shifted peak and distribution for caudal thalamic explants growing on *Lhx2* mutant ventral telencephalic preparations (i; $p<0.0001$).

striking contrast, the direction of caudal thalamic projections was significantly altered (Figs. 6g–i; $p<0.0001$). Indeed, the quantitation curve representing this caudal set appears comparable to that of the middle set in control explants (compare the peaks of the curves in Figs. 6f and i). Therefore, there is a specific perturbation in guidance cues in the ventral telencephalon that is restricted to the pathfinding of *caudal* thalamic fibers, such that they grow in a medialized direction.

To probe for possible causes underlying this defective directionality, we examined the *Lhx2* mutant ventral telencephalon for markers of patterning and pathfinding. Previous studies have established that the overall morphology and the specification of several discrete structures appear normal (Bulchand et al., 2001; Vyas et al., 2003). For example, at E12.5, the mantle of the LGE and MGE expresses *Dlx2*, and the LGE and septum express *Isl1* in patterns that are comparable with control brains (Figs. 7a–d; Bulchand et al., 2001; Vyas et al., 2003). At E14.5, expression of *Netrin-1*, a guidance molecule known to be an attractant for thalamocortical axons (Braisted et al., 2000), also appears normal in the absence of Lhx2 (Figs. 7e, f). Another important group of axon guidance molecules is the Semaphorin family, known to be repulsive to axons in several systems (Bagnard et al., 1998; Chedotal et al., 1998). In the absence of Sema6A, caudal thalamic projections are selectively perturbed (Leighton et al., 2001). We found *Sema6A* expression to be strikingly upregulated in *Lhx2* mutant brains. When examined at several rostrocaudal levels, a more restricted upregulation is seen at rostral to mid-levels, whereas

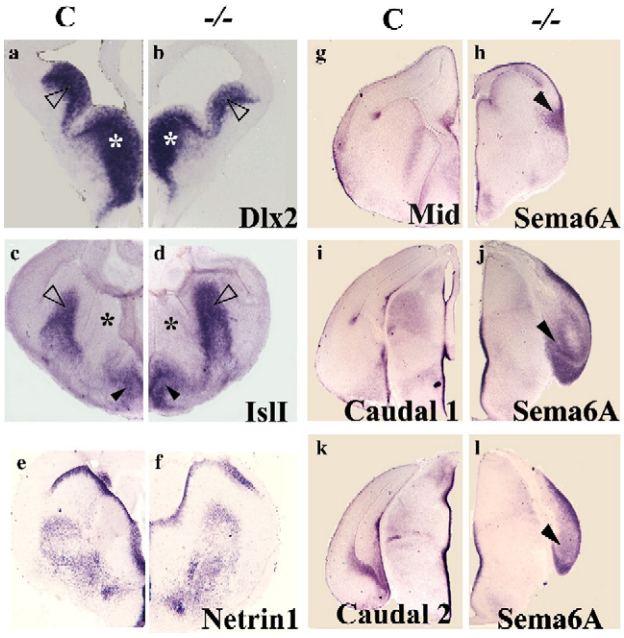


Fig. 7. Expression of ventral telencephalic guidance molecules in Lhx2−/− brains. At E12.5, *Dlx2* expression (a, b) is seen in the LGE (open arrowhead) and MGE (asterisk), while *Isl1* (c, d) marks the LGE (open arrowhead) and the septum (arrowhead) in both control and mutant sections. At E14.5, *Netrin1* expression identifies restricted cell domains in the ventral telencephalon of both control and mutant sections. *Sema6A* expression is in limited regions of control sections (g, i, k), but appears in an ectopic patch in the lateral region of mid-level mutant sections (arrowhead, h) and is vastly expanded in caudal sections of *Lhx2* mutant brains (arrowhead, j, l).

at caudal levels the entire ventral telencephalon displays aberrant upregulation (Figs. 7g–l). This may provide a possible basis for the defective topography of control caudal thalamic projections when growing on *Lhx2* mutant ventral telencephalic preparations.

Discussion

Several surprising findings emerge from our analysis of the thalamocortical pathway in the *Lhx2* mutant. First, our results argue against a requirement for Lhx2 as part of a combinatorial code for patterning the dorsal thalamus at early stages, or for cell-autonomously regulating the topography of thalamic projections when the tract is traversing the ventral telencephalon. We find, however, an unexpected role for Lhx2 in the ventral telencephalon, involving the development of internal capsule cells and also in selectively guiding the topography of caudal, but not rostral or middle thalamic projections.

Patterning the dorsal thalamus

A detailed analysis of the E12.5 and E14.5 dorsal thalamus reveals normal regional patterning in the absence of Lhx2, though there may be defects that do not reveal themselves in the embryonic stages examined. Since the thalamocortical tract has normally reached the cortex by E14.5, however, any late-appearing defects in the dorsal thalamus would not affect the growth of the tract into the ventral telencephalon or its topography while crossing this region. Therefore we looked for other causes that may underlie the two phenotypes we observed, failure of entry into the ventral telencephalon *in vivo* and abnormal topography of caudal thalamic projections *in vitro*.

A severe depletion of IC pioneer cells may underlie the mutant thalamocortical pathfinding defect

Despite the shrunken morphology of the *Lhx2* mutant ventral telencephalon, we find many molecular and cellular features relevant to thalamocortical tract pathfinding to be normal in the absence of Lhx2. The early defect in the *Lhx2* mutant thalamocortical tract correlates with defective development of IC cells, such that only a few of these cells are detected by backlabeling from the dorsal thalamus. These cells are proposed to perform a pioneer function by sending projections to the dorsal thalamus early in development, to guide thalamocortical axons across the DTB (Mitrofanis and Guillery, 1993; Molnar et al., 1998a,b; Molnar and Cordery, 1999). There are as yet only correlative data supporting such a role: in the absence of Mash1, the IC pioneers appear to be missing, and the thalamocortical tract fails to enter the ventral telencephalon (Tuttle et al., 1999), similar to the defect we report in the *Lhx2* mutant. In the *Emx2* mutant, where these pioneer cells are displaced ventrally, thalamocortical axons initially project in aberrant, ventral directions after entering the telencephalon, before later correcting their trajectories and innervating the cortex (Lopez-Bendito et al., 2002). Together, these findings support the idea that the

DTB, a relatively small junctional structure between the diencephalon and telencephalon, poses a pathfinding challenge that the thalamocortical tract may need assistance in crossing, *in vivo*. In contrast, when dorsal thalamic explants are juxtaposed directly with ventral telencephalic preparations *in vitro*, the requirement of IC pioneers is apparently not critical. Instead of having to navigate via the ventral thalamus and then turn to find the junctional tissue of the DTB, *in vitro*, dorsal thalamic axons can access the ventral telencephalon easily across the juxtaposed interface of the respective explants.

Even though the evidence supporting the function of IC cells is only correlative thus far, it is worth contrasting this hypothesized role with that demonstrated for the newly discovered population of corridor cells (Lopez-Bendito et al., 2006). This study showed that growth of thalamocortical axons across the ventral telencephalon critically requires the corridor cells. However, the corridor cells were not tested specifically for their ability to aid the crossing of the DTB since dorsal thalamic explants were placed directly onto ventral telencephalic slices, from which ectopic location the thalamocortical axons grew via the "corridor" (Lopez-Bendito et al., 2006). The *Lhx2* mutant presents an interesting contrast to the *Mash1* mutant, in which both the IC cells (Tuttle et al., 1999) as well as the corridor cells (Lopez-Bendito et al., 2006) are missing, and the thalamocortical tract also fails to enter the ventral telencephalon. Our findings suggest that the presence of corridor cells in the *Lhx2* mutant may not be sufficient for the *entry* of the thalamocortical tract into the ventral telencephalon *in vivo*, but the lack of IC pioneers may be critical. IC pioneers appear to not be necessary for growth of the thalamocortical tract *across* the ventral telencephalon, however, either in the *Lhx2* mutant explant cultures (this study) or in slice cultures of *Mash1* mutant tissue. Lopez-Bendito et al. (2006) demonstrated that thalamocortical axons are able to grow in *Mash1* mutant slice cultures, despite the absence of IC cells, if corridor cells are transplanted from normal donor explants.

Therefore a 2-step cellular mechanism may be operational: IC cells proposed as a pioneer population that guide the initial entry of dorsal thalamic axons across the DTB *in vivo*, and corridor cells that are known to be crucial for their growth across the ventral telencephalon. In the *Lhx2* mutant, which has a severe depletion of IC cells, the thalamocortical tract is unable to enter the ventral telencephalon. In explant culture, however, the thalamocortical tract is not only able to enter, but also grows well across the ventral telencephalon, a phenomenon which requires the presence of the permissive corridor.

A role for Lhx2 in sorting thalamic projections

When dorsal thalamic axons enter the ventral telencephalon they are "sorted" prior to their entering the cortex, and this initial sorting is thought to lay out the topography of inter-areal cortical projections (Garel et al., 2002; Vanderhaeghen and Polleux, 2004). Thalamic nuclei are located at distinct positions along the rostrocaudal and medio-lateral axis of the thalamus, and this arrangement may itself produce an ordered set of projections even before the thalamocortical tract exits the thalamus. The tract fans out upon entering ventral telencephalic territory, where cell-autonomous and non-autonomous mechanisms would be expected to interact to "sort" the tract prior to its entering the cortex. *In vivo*, rostro-medial thalamic nuclei connect to rostral cortical parts whereas caudo-lateral nuclei innervate more caudal cortical areas. In the assay established by Seibt et al. (2003), thalamic explants are separated based on their rostrocaudal positions. This assay has been criticized as not fully representative of the *in vivo* picture, since the medio-lateral aspect of the organization of thalamic nuclei is not reflected in this division of explants along the rostrocaudal axis (Garel and Rubenstein, 2004). Despite this limitation, this assay elegantly demonstrated a cell-autonomous role for bHLH transcription factor Ngn2 in directing the topography of axons from rostral explants of the dorsal thalamus (Seibt et al., 2003). Lhx2 has also been proposed to play a cell-autonomous role, based on its expression in the dorsal thalamus (Nakagawa and O'Leary, 2001). Therefore, we used the established assay without introducing any further parameters so that our results may be better compared with those from the *Ngn2* mutant.

We find, surprisingly, that Lhx2 is not required in the dorsal thalamus, but rather in the ventral telencephalon, for the correct guidance of caudal thalamic projections. Lhx2 may act by misregulating critical guidance molecules in the ventral telencephalon. Sema6A has been shown to be an important player in thalamocortical guidance. In the Sema6A mutant, the thalamocortical axons display aberrant trajectories into regions they normally do not enter (Leighton et al., 2001). Significantly, it is the *caudal* projections that are misguided in the *Sema6A* mutant (Leighton et al., 2001). Furthermore, in the *Ebf1* mutant, *Sema6A* expression in the ventral telencephalon is greatly reduced, and thalamocortical axons enter areas that are normally *Sema6A* positive in the wildtype (Garel et al., 2002). *Lhx2* mutant ventral telencephalic explants may exhibit an opposite scenario, in which the ventral telencephalon is extraordinarily repulsive to caudally headed thalamic axons, which instead grow more medially. Taken together, the studies suggest that this caudal subset of thalamic afferents is sensitive to levels of *Sema6A* expression.

Normal sorting of rostral and middle thalamocortical projections in the absence of corticothalamic axons

Cortical inter-areal topography is thought to be laid out by the ordered positioning of thalamocortical fibers in the ventral telencephalon, in advance of their entering the cortex (Garel et al., 2002). Interaction with corticothalamic axons (the "Handshake hypothesis," Molnar and Blakemore, 1995) is one proposed mechanism for sorting the thalamocortical projections while they are growing across the ventral telencephalon. The absence of neocortex in the *Lhx2* mutant (Bulchand et al., 2001; Fig. 1) thus opens another major angle to analyzing thalamocortical tract guidance. Our *in vitro* studies reveal that control thalamic axons from the rostral and middle portions of the thalamus are indeed capable of growing correctly, in extent and direction within the *Lhx2* mutant ventral telencephalon, which

despite the absence of neocortex is capable of producing the necessary cues to guide them.

Exploring the role of Lhx2 in the dorsal thalamus

An interesting question that still remains unanswered is the function of Lhx2 in the dorsal thalamus. Lhx9, which is closely related to Lhx2 (Retaux et al., 1999) and shows overlapping expression in the embryonic dorsal thalamus (Nakagawa and O'Leary, 2001; Bulchand et al., 2003), may possibly substitute for Lhx2 in controlling dorsal thalamic patterning or the topography of thalamic projections at the stages we examined. This model would imply a redundancy between Lhx2 and Lhx9, rather than a combinatorial code where both molecules are required, as has been proposed for these proteins (Nakagawa and O'Leary, 2001). Alternatively, Lhx2 may be required for the topography of the thalamocortical tract, but only at later stages, after it has entered the cortex, an angle that is worthwhile to explore in future studies, using more selective reagents such as conditional knockout mice.

A different scenario is that Lhx2 is genuinely not required to perform developmental functions such as cell fate specification or pathfinding in dorsal thalamic neurons. Recent studies have shown that *Lhx2* displays activity dependent regulation in the hippocampus (Elliott et al., 2003), suggestive of a role in mediating other aspects of neuronal function (Hevroni et al., 1998). Thus, *Lhx2* could regulate the expression or synthesis of neurotransmitters, receptors, or neuropeptides. Indeed, the *Drosophila* homolog of Lhx2, Apterous, controls expression of the neuropeptide FMRFamide in a specific subset of neurons in the ventral nerve cord (Allan et al., 2003).

In summary, we find that Lhx2 is required for crucial early steps in the normal development of the thalamocortical tract, and there is a critical requirement for Lhx2 in the ventral telencephalon for the topographic growth of a subset of thalamic projections. This is the first report of a role for a LIM–HD gene in the emerging complex picture of thalamocortical pathfinding.

Acknowledgments

We thank D. Anderson, S. Garel, J. Johnson, M. Tessier-Lavigne, J. Nathans, C. W. Ragsdale, J. L. Rubenstein, and V. Pachnis for gifts of plasmid DNA, and F. D. Porter for gifting us *Lhx2* breeding pairs. We also thank Franck Polleux for generous advice on the in vitro explant culture assay, Elizabeth Grove, Ben Martynoga, Amrita Nair and Madhurima Benekreddy for critical input, and the animal house staff of NCBS and TIFR for breeding the Lhx2-GFP mice. This work was supported by a Wellcome Trust Senior Fellowship (056684/Z/99/Z) and a Swarnajayanti Fellowship (Dept. of Science and Technology, Govt. of India) to ST.

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